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BRIEF COMMUNICATION

Electroporation as a Tool to Study In Vivo Spinal Cord Regeneration

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Tailed amphibians such as axolotls and newts have the unique ability to fully regenerate a functional spinal cord throughout life. Where the cells come from and how they form the new structure is still poorly understood. Here, we describe the development of a technique that allows the visualization of cells in the living animal during spinal cord regeneration. A microelectrode needle is inserted into the lumen of the spinal cord and short rapid pulses are applied to transfer the plasmids encoding the green or red fluorescent proteins into ependymal cells close to the plane of amputation. The use of small, transparent axolotls permits imaging with epifluorescence and differential interference contrast microscopy to track the transfected cells as they contribute to the spinal cord. This technique promises to be useful in understanding how neural progenitors are recruited to the regenerating spinal cord and opens up the possibility of testing gene function during this process. *Developmental Dynamics 226:418–425, 2003.*

Key words: spinal cord; electroporation; regeneration

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INTRODUCTION

Spinal cord regeneration is one of the most amazing but least understood characteristics of the axolotl. How neural progenitors are recruited from the mature tissue to build the new spinal cord is poorly understood. Regeneration of the spinal cord is most easily studied in the context of tail regeneration. After tail amputation, the end is sealed by migration of epidermis over the wound. Subsequently, a mound of proliferating undifferentiated mesenchymal cells, called the blastema, is formed by means of dedifferentiation of mature cells (Echeverri et al., 2001). The blastema then grows and eventually differentiates to form the lost structures (Iten and Bryant, 1976a,b; Chernoff, 1996; Clarke and Ferretti, 1998). At the same time that the mesenchymal blastema is forming, the spinal cord is regrowing as a separate entity. Ultrastructural studies indicate that the cells at the end of the spinal cord undergo an epithelial-to-mesenchymal transition that is consistent with the induction of cell migration (O'Hara et al., 1992). The cells then seal over the end of the spinal cord, and at 4–6 days, a bulbous structure is visible called the terminal vesicle, the function of which remains unclear (Holtzer, 1956; Egar and Singer, 1972; Nordlander and Singer, 1978). The ependymal tube that contains the dividing neuroepithelial precursors then grows in length through cell division. After several weeks, differentiation of new neural ganglia is observed to occur in a rostral-to-caudal sequence (Nordlander and Singer, 1978; Singer et al., 1979; Geraudie et al., 1988).

Past studies have suggested that the first events in spinal cord regeneration are the induction of proliferation and migration of the cells close to the plane of amputation (Holtzer, 1956; Butler and Ward, 1967; Nordlander and Singer, 1978; Egar, 1972; Arsanto et al., 1992; Benraiss et al., 1999). It is thought that the ependymal cells lining the neural canal can generate all the cell types to form a new fully functional spinal cord, suggesting that, in urodeles, ependymal cells retain or acquire upon injury the characteristics of embryonic neuroepithelial

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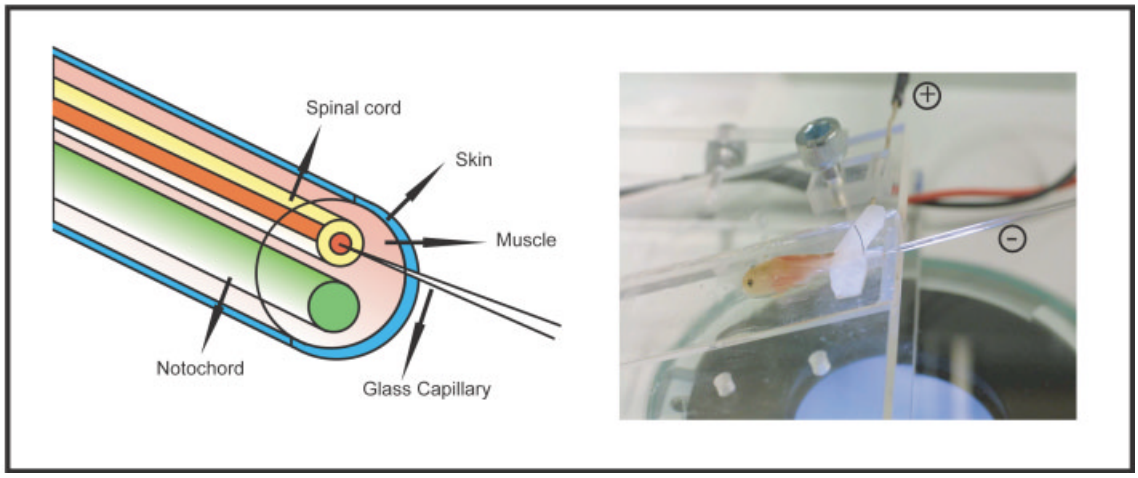

Fig. 1. Schematic diagram of microelectrode electroporation set-up and a photo of the experimental set-up. First the tail is amputated, a glass micropipette needle electrode containing the DNA solution and the negative wire electrode is then inserted into the spinal cord, and a pulse of 50 V, 200 Hz, and pulse length of 100 msec was applied five times.

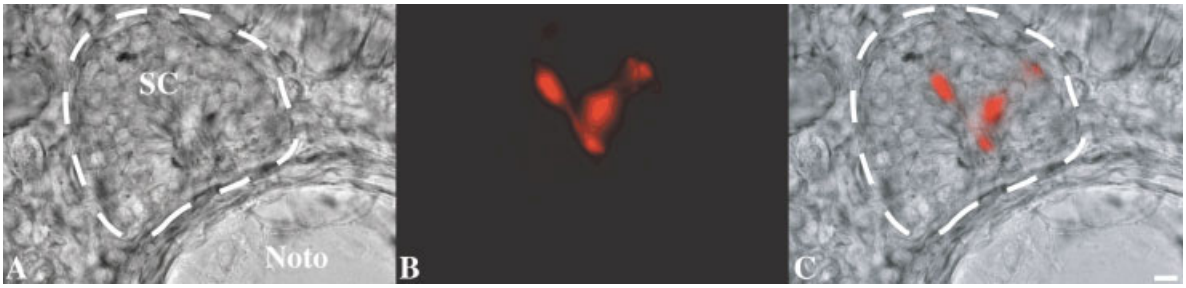

Fig. 2. Shown are 100-μm transverse cryosections of the regenerating axolotl tail. **A:** Differential interference contrast (DIC) image of the spinal cord (SC) and part of the notochord (Noto). **B:** A fluorescent image of cells labeled by single-cell electroporation with CMV-DsRed2-N1. **C:** Overlay of DIC image and the fluorescent image, showing that the labeled cells are cells of the spinal cord. Scale bar = 50 μm in C (applies to A–C).

cells (see Chernoff, 1996; Clarke and Ferretti, 1998).

It is not known, however, how many of the spinal cord cells behind the amputation plane are induced to participate in regeneration. Furthermore, it is unknown what diversity of cell types a single progenitor cell forms. To address such issues, Benraiss et al. used biolistics to transfect the human alkaline phosphatase gene DNA into spinal cord cells at the plane of amputation and could later identify cells positive for the marker outside of the spinal cord, mainly in Schwann cells and melanocytes (Benraiss et al., 1996). Their data indicated that the cells probably left the spinal cord from the dorsal side of the terminal vesicle, but their studies were limited by the inability to actually visualize the movement of the cells. However, this technique was not successful in labeling cells that would contribute to central nervous system structures.

Here, we describe how microelectrode-mediated electroporation can be used to precisely and efficiently label spinal cord cells in vivo, allowing them to be followed in live animals during the course of regeneration. Electroporation is a well-established method for introducing charged molecules like DNA, RNA, dyes, and proteins into cells (Neumann et al., 1982; Potter, 1988). Electroporation works by the application of electrical pulses of short duration to form temporary pores in the cell membrane allowing charged macromolecules like DNA to actively enter the cell. Noncharged molecules may also enter cells by passive diffusion with this method (Neumann et al., 1982). Efficient, noninvasive electroporation in vivo was achieved by the implementation of a rapid series of short pulses (Muramatsu et al., 1996). Since then, in ovo electroporation has become a technique widely used by chick embryologists (Itasaki et al., 1999; Swartz et al., 2001) and extensively applied to other systems (Saito and Nakaatsuji, 2001; Tawk et al., 2002). Traditionally, macroscopic flat or tweezer electrodes were used to generate populations of labeled cells. Recently, a micropipette electrode technique was used to target transfection to single neurons within the intact brain of live *Xenopus* (Haas et al., 2001). Here, we describe how this technique has been adapted to transfect single cells or small groups of cells within the spinal cord of an axolotl close to the plane of amputation that then allows the labeled spinal cord cells to be followed during the course of regeneration.

TABLE 1. Effect of Electroporation Parameters on Transfection Efficiency[a]

Voltage (V)	Pulse length (msec)	Number of pulses	Trains (50 Hz, 1 sec)	Trains (200 Hz, 1 sec)	No. of labelled animals	Number of animals
A						
20	1	5			1	100
20	100	5			2	100
50	1	5			5	100
50	100	5			6	60
B						
50	10		5		9	60
50	100		5		6	60
50	10			5	9	60
50	100			5	18	60
80	10			5	1	25

[a]Animals were electroporated in the spinal cord as described in the text and checked for transfected cells after 2 days. A: Electroporation was performed with a BTX Electro Square Porator 830. B: Electroporation was performed with a Grass SD9 stimulator.

RESULTS

Labeling Spinal Cord Cells by Means of Electroporation

Our goal was to develop a technique to label single or a few ependymal cells in the spinal cord and track their behavior in live animals during regeneration. Axolotls of 2–3 cm were used because regeneration is rapid and the tail tissue is transparent, allowing good visualization of the spinal cord. Micropipette electroporation was carried out by amputating the tail and then inserting the glass capillary electrode containing the plasmid DNA into the neural canal through the cut end of the spinal cord (Fig. 1). By tilting the axolotl at an angle as shown in Figure 1 we could easily insert the needle into the lumen of the spinal cord under the stereomicroscope.

Electroporation could be achieved by using a glass electrode (tip diameter of 1–2 μm, resistance of 10–15 MΩ) connected either to a conventional square pulse generator or a Grass stimulator. As seen in Table 1, the highest efficiency was achieved by using the stimulator to generate five trains of pulses at 50 V and 200 Hz, 100-msec pulse length. With these parameters, 30% of animals contained labeled cells. Animals with labeled cells contained an average of three labeled cells. Application of voltages greater than 50 V led to obvious disruption of the tissue around the electrode, and 80 V, the highest voltage tested, did not yield a higher transfection efficiency (Table 1). Although the axolotls could recover from such tissue disruption to regenerate normal tissue (data not shown), we chose not to pursue such conditions.

Transfection of cells with conventional green and red fluorescent protein (GFP and DSRed2) plasmids (see Experimental Procedures section for details) resulted in efficient expression of fluorescent protein. We found that nuclear GFP driven by the cytomegalovirus (CMV) promoter was first visible after 12–24 hr, whereas DsRed2 under the same promoter became visible at 36–48 hr due to the longer maturation time for the protein. Bright GFP fluorescence could be detected for 7–14 days, whereas DsRed2 lasted 14–22 days. The length of visualization appears to be lost more quickly in cells undergoing rapid cell division.

Transfected Cells Lie Within the Spinal Cord

The use of ubiquitous promoters means that cells outside the spinal cord can theoretically also be transfected. The specificity of the transfection technique depends on the optical clarity of the axolotl tail, allowing us to visualize the diameter and length of the spinal cord as the micropipette is being inserted into the spinal cord. To assess the accuracy of our technique, we cryostat sectioned the transfected tail 2 or 4 days after amputation and found that, in all tail samples that were cryosectioned (n = 12), the cells expressing the GFP or DsRed proteins lay within the spinal cord (Fig. 2).

Electroporated Cells Faithfully Contribute to the Regenerating Spinal Cord

We found that the electroporated tail tissue underwent normal regeneration, and transfected cells contributed to the regenerating structure. Tail regeneration in 2- to 3-cm axolotls occurs in approximately 14 days, at which point blastema cells begin differentiating into structures such as the rod of cartilage ventral to the spinal cord (Echeverri et al., 2001).

To follow cells during regeneration, animals were anesthetized each day, and the cells were imaged by using a 10× or 20× Plan-Neofluor objective both with fluorescence optics to document the cell, and with DIC optics to localize the position of the cell within the regenerating tissue. In axolotls, the amputation plane can always be identified as the point where the notochord ends abruptly, because the regenerated portion of the tail forms cartilage instead of notochord (Holtzer, 1956). This point also represents the point at which mature, striated muscle fibers also terminate (Echeverri et al., 2001).

Although most animals contain several transfected cells, it is possible to follow the fate of individual cells with this protocol with relative certainty. The cell cycle in axolotl cells lasts 72 hr, so the position and number of cells changes slowly enough that documentation every 24 hr allows individual cells to be followed. Figure 3 illustrates a typical example where two cells in the spinal cord were cotransfected with the CMV-nuclear GFP and cytoplasmic CMV-DsRed2 plasmids, and these cells contribute to the growing ependymal tube. Two days after amputation and electroporation, the cells

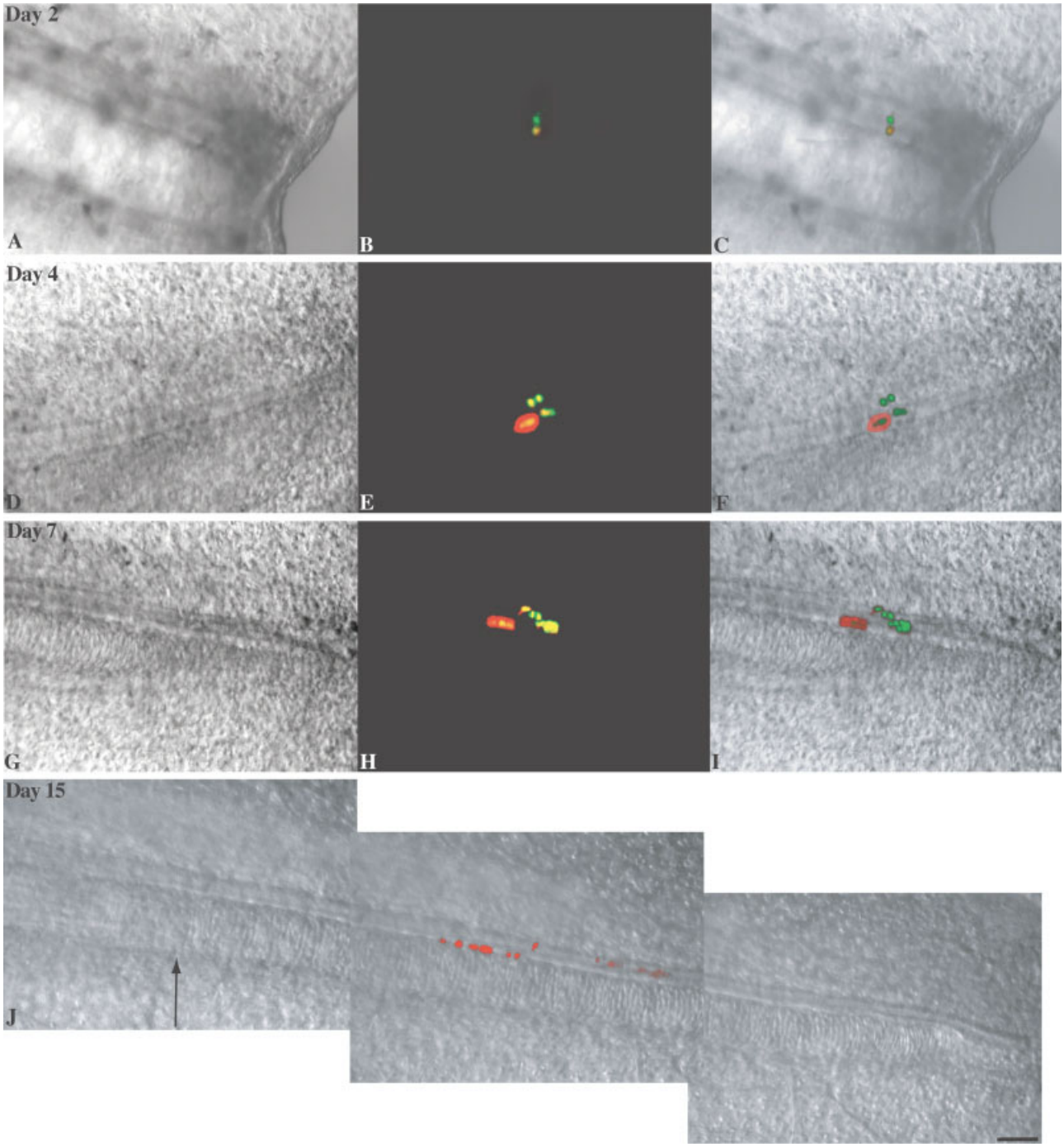

Fig. 3. A–J: Cells were transfected with cytoplasmic DsRed2-N1 and nuclear green fluorescent protein plasmids (B,C). The fluorescent overlay with differential interference contrast (DIC) image at 2 days after amputation shows both cells lying within the spinal cord approximately 250–300 μm away from the plane of amputation (C). Over the following 2 days, the cells divide and contribute to the regenerating spinal cord shown in E,F. (In these panels, only the regenerating portion of the tissue is visible.) The cells continue to divide rapidly as the new spinal cord is growing (G–J). J: Composite of the DIC images overlaid with the fluorescent image at 15 days. At this point, the original two cells have given rise to approximately 10 cells on both the dorsal and ventral sides of the midportion of the new spinal cord. These clusters span a distance of 560 μm along the anterior/posterior axis. The arrow indicates the original plane of amputation. Scale bar = 100 μm in J (applies to A–J).

are lying approximately 300 μm away from the plane of amputation (Fig. 3C). GFP is visible in both cells, whereas the DsRed2 is visible in the cell lying in the ventral spinal cord but not the cell in the dorsal side (Fig. 3B). The profile of DsRed2 fluorescence becomes brighter over the next days. By day 4, the regenerating spinal cord has grown to 600 μm in length (Fig. 3D) and both cells have moved out of the mature tissue

and divided to form part of the regenerating spinal cord (Fig. 3E,F). The labeled cells increase in number again by day 7 to give rise to a cluster of cells within the regenerating ependymal tube that spans a distance of 190 μm along the anteroposterior (A/P) axis. By day 15 of regeneration when the spinal cord has grown to a length of 2 mm, the cells form an elongated clone of cells spanning 560 μm along the A/P axis. At this time, the GFP was no longer visible but the DsRed protein was still visible (Fig. 3J). In summary, the cells that were initially 300 μm behind the plane of amputation have proliferated and migrated to give rise to cells on both the dorsal and ventral sides of a segment of the midportion of the newly formed spinal cord. Neither cell has left a descendent in the mature spinal cord (Fig. 3J).

Fifteen animals contained labeled cells that lay within 400 μm of the plane of amputation. The labeled cells from 11 of these animals came out and contributed to the regenerating spinal cord, whereas in four animals, the labeled cells remained at the plane of amputation. The cells that moved out into the spinal cord gave rise to an average of 10 visible descendants. The ultimate fate of the labeled cells during regeneration is published in a separate report (Echeverri and Tanaka, 2002).

Electroporation Can Be Used to Study the Fate of Different Cell Types

In Figure 3, we showed that electroporation can be used to transfect the ependymal cells in the mature spinal cord that participate in spinal cord regeneration. Figure 4 demonstrates that it can also be used to transfect neurons (Fig. 4B,C). In the same animal, an ependymal cell lying close to the plane of amputation has also been labeled (Fig. 4B,C), so here we can follow the fate of two cells approximately 250 μm apart. Over time, the neuron remains unchanged but the cell on the right begins to divide (Fig. 4E,F). These cells divide again and also migrate into the regenerating portion of the spinal cord (Fig. 4H,I). By day 10 of regeneration, the initial cell has given rise to approximately 10 cells, which now spread out to populate the ventral side of the newly forming regenerate (Fig. 4J,K).

Previous lineage studies have shown that mature differentiated muscle fibers dedifferentiate during regeneration and form mononucleate cells that populate the blastema (Lo et al., 1993; Kumar et al., 2000; Echeverri et al., 2001). It was unknown whether differentiated neurons could also dedifferentiate during regeneration. In our experiments to date, six labeled neurons, identified by morphology, did not dedifferentiate or migrate into the regenerate. Such behavior is illustrated in Figure 3. The left-most cell displays the characteristic process of a differentiated neuron (Fig. 3C). Although this cell changes shape slightly during regeneration, it retains its process throughout regeneration, does not divide, and it does not migrate into the regenerate (Fig. 3F–J). In the future, the use of neuronal-specific promoters driving GFP will allow us to definitively address whether or not neurons dedifferentiate during regeneration.

DISCUSSION

New Method for Tracking Cells During Spinal Cord Regeneration

In this report, we describe a method to transfect DNA into the axolotl spinal cord by means of electroporation with a DNA-filled microelectrode. A key aspect of targeting transfection to the neural precursors is the ability to insert the microelectrode into the lumen of the spinal cord. Coupled with the use of small, optically clear animals, it is possible to visualize the labeled cells over time as they participate in forming the regenerated spinal cord. As exemplified in Figure 3, during the growth of the regenerating spinal cord, we observe recruitment of progenitor cells from mature tissue into the regenerating structure (day 2–4), cell division (day 4–15), and spreading of the clones along the A/P axis (day 7–15).

None of the six neurons we have labeled was observed to dedifferentiate. These preliminary data are consistent with those of Zhang et al. (2003) who retrogradely labeled spinal cord neurons with rhodamine dextran and tracked the fate of over 60 neurons during spinal cord regeneration in *Pleurodeles waltl*. Zhang et al. additionally saw no evidence of bromodeoxyuridine incorporation in neuronal cell bodies close to the amputation plane. Taken together, these results indicate that, during regeneration, some mature cell types such as muscle undergo dedifferentiation, whereas others such as neurons do not.

Future Improvements and Prospects for the Electroporation Method

Haas et al. originally reported electroporation of single neurons in the *Xenopus* brain by using very similar electroporation conditions, with a success rate of 30% in terms of electroporation attempts (Haas et al., 2001). Although we observe a similar overall success rate, we label on average three cells around the needle tip. It is not yet known if this difference stems from differences in cell physiology or a difference in the electroporation configuration. Haas was electroporating differentiated neurons, whereas we are electroporating cells that have been stimulated to divide. An additional consideration is that Haas pierced the brain tissue with the microelectrode at a very steep angle. In contrast, we insert our electrode up to 400 μm into the end of the open neural canal. We are currently exploring further electroporation conditions that will allow us to label a single cell at relatively high efficiency. This method would allow unambiguous cell fate tracking studies of neural progenitors in the spinal cord. A second consideration for cell fate studies will be the duration of expression. Electroporation results in transient transfection where expression lasts, in axolotls, up to 22 days, depending on the cell type transfected and the expression construct chosen (K. Echeverri, E.M. Tanaka, unpublished data). Although this length of time may be sufficient to

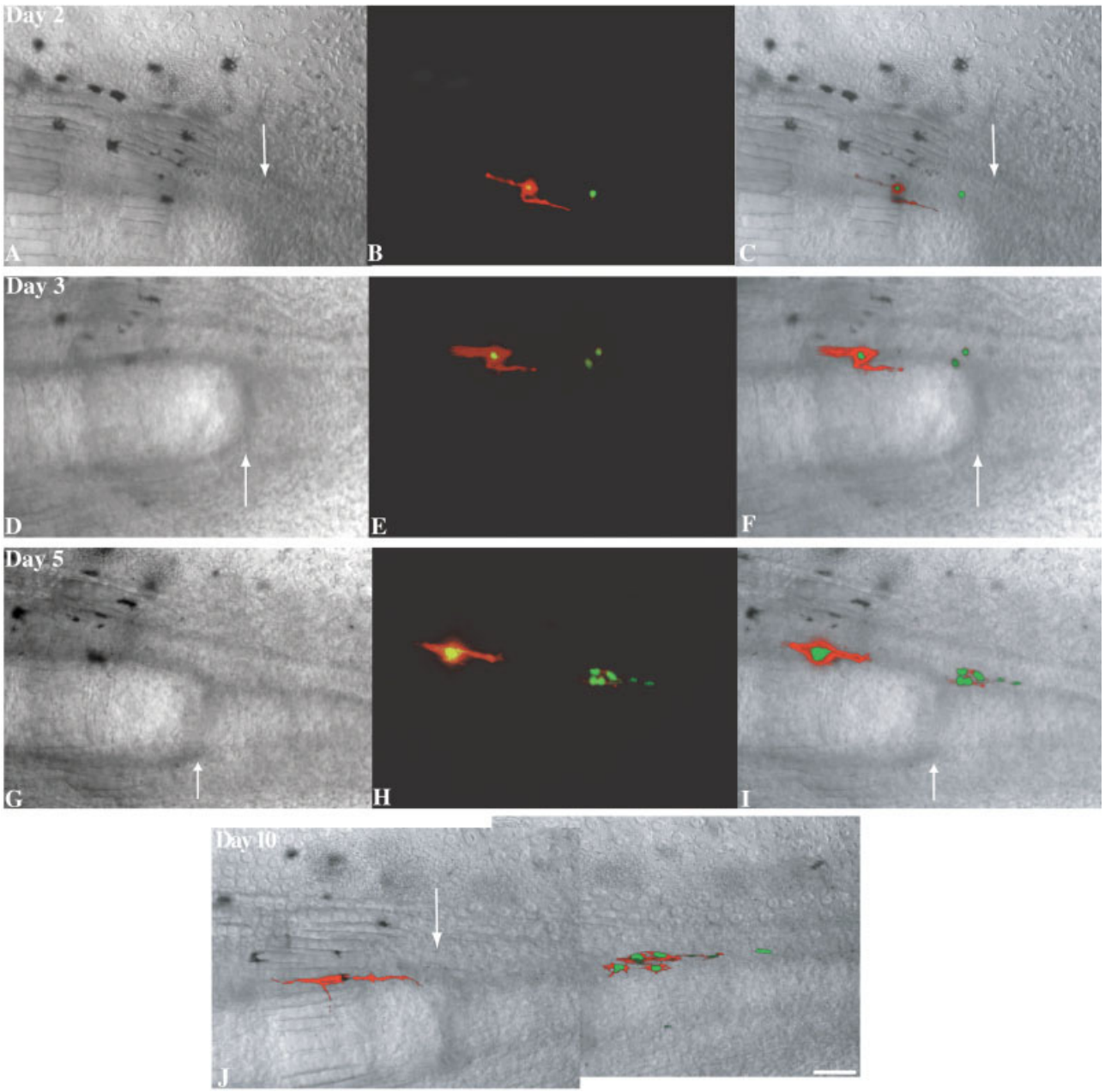

Fig. 4. A–J: Ependymal cells and neurons can be transfected by using electroporation. B,C: Each cell type expressing the DsRed and nuclear green fluorescent protein 2 days after electroporation. As regeneration progresses, the ependymal cells divide rapidly (E,H), whereas the neuron remains unchanged (E,H). By 10 days after amputation, the rapidly dividing ependymal cells have migrated out to contribute to the regenerating spinal cord (J), whereas the neuron remains behind the amputation plane putting out processes to probably innervate the muscle lying above it. The arrows indicate the original plane of amputation. Scale bar = 100 μm in J (applies to A–J).

follow the early events of differentiation during regeneration, it does not allow late differentiative events to be observed. Again, further modification of plasmid constructs or electroporation conditions may be required.

Given the efficiency of this method to deliver DNA plasmids into cells within the spinal cord and also into other cell types of the tail such as muscle and dermis (K. Echeverri, E.M. Tanaka, unpublished data), we imagine that this technique can be further developed to deliver other charged macromolecules like proteins or siRNAs into specific cell types to allow us to further elucidate the molecular signaling mechanisms underlying regeneration.

EXPERIMENTAL PROCEDURES

Animals and Surgery

These experiments were carried out by using larval *Ambystoma mexicanum* (axolotls) bred in captivity in our

facility. Axolotls were maintained at 17°C in 40% Holtfreter's and fed daily with artemia. All experiments were carried out on axolotls anesthetized in 0.01% ethyl-p-aminobenzoate (Sigma).

Tail Amputation and Electroporation

Anesthetized animals were immobilised on an optically clear polymer matrix:sylgard (Dow Corning, Wiesbaden, Germany), and the tail was amputated under an Olympus Stereo SZ microscope. A kimwipe soaked in phosphate buffered saline (PBS) was placed over the axolotl and the ground electrode lying next to the animal (Fig. 1). The micropipettes were pulled by using a Sutter Flaming Brown P-97 puller to a tip size of approximately 1–2 μm. A silver wire was placed inside the micropipette containing 0.5 μg/μl of plasmid DNA purified by using a Quiagen Maxi Prep kit. This apparatus was inserted into the neural tube, and five trains of pulses of 50 V, 200 Hz, and pulse length 100 msec were applied by using a SD9 Stimulator (Grass Telefactor, USA). The animals were then allowed to recover in 40% Holtfreter's containing Pen/Strep.

All DNA plasmids used were derived from the Clontech Living Colours collection, and maps are available from Clontech (http://www.clontech.com). In the studies described here, we used DSRed2-N1 and eGFPN2, where eukaryotic gene expression is driven by the cytomegalovirus promoter. The nuclear eGFP plasmid was a kind gift from Wulf Haubensak (Max-Planck Institute, Dresden, Germany) and was made by insertion of the sequence encoding the SV40 nuclear localization signal at the C-terminus of the eGFP molecule.

Imaging of Labeled Cells

Animals containing labeled cells were imaged every day by anesthetizing the animals in 0.01% ethyl-p-aminobenzoate (Sigma), placing them on a coverslip and imaging by using a 10× or 20× Plan-Neofluor objective on a Zeiss Axiovert 2 microscope controlled by a Metamorph image acquisition system (Visitron, Munich, Germany). The pigment cells on the dorsal side of the spinal cord were used as an aid for correct orientation.

Cryostat Sectioning

Two or 4 days after electroporation, tails containing labeled cells were fixed in 4% paraformaldehyde containing 5% sucrose at room temperature for 20 min. They were then washed 3 × 5 min in PBS + 5% sucrose, followed by 4 × 20 min washes in the same wash solution.

The tails were then embedded in 1.5% agarose plus 5% sucrose. The agarose was allowed to set, and then the blocks containing the tails were cut out and placed in a solution of 30% sucrose overnight at 4°C.

For sectioning, the agarose blocks were embedded in Tissue Tec (Sakura), and the cryosections were collected on Histobond Adhesion microslides (Marienfeld). The sections were allowed to air dry for 2–3 hr and were then covered with glycerol and a coverslip placed on top. The samples were then imaged by using a Zeiss Axiovert 2 microscope controlled by a Metamorph image acquisition system (Visitron, Germany).

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